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CTEN/tensin 4 expression induces sensitivity to paclitaxel in prostate cancer

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For Peer Review

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ABSTRACT

BACKGROUND. Recently, we established paclitaxel-resistant prostate cancer cell lines (PC-3-TxR and DU145-TxR). [To determine](#) the mechanisms of paclitaxel resistance in PC-3-TxR cells, we compared the gene expression [profiles](#) between PC-3 and PC-3-TxR cells. [Our results indicated](#) that expression of the CTEN (C-terminal tensin like protein, tensin 4) gene was down-regulated by 10-fold in PC-3-TxR cells. We investigated the possibility that CTEN overexpression restores paclitaxel sensitivity.

METHODS. We investigated how knockdown [and](#) overexpression of CTEN in androgen-independent cell lines affect paclitaxel sensitivity by colony formation assay and growth inhibition assay. To [determine the](#) mechanisms by which CTEN affects paclitaxel sensitivity, we investigated the [relationships](#) between CTEN and F-actin or EGFR in PC-3 cells. We also examined [the](#) association between expression of CTEN and grade of prostate cancer by immunohistochemistry using tissue microarray [analysis](#).

RESULTS. Down-regulation of CTEN, which [is located](#) in [the](#) cytoskeleton, played an important role in paclitaxel resistance in PC-3-TxR cells. Knockdown of CTEN expression in PC-3 cells induced paclitaxel resistance. Overexpression of CTEN in PC-3-TxR and DU145-TxR cells restored paclitaxel sensitivity. CTEN expression was inversely correlated with F-actin and EGFR expression. Then

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7 knockdown of actin and EGFR in PC-3-TxR cells recovered paclitaxel sensitivity, indicating [that](#) CTEN
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10 down-regulation mediates paclitaxel resistance through elevation of EGFR and actin expression.
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13 Moreover, CTEN expression was inversely correlated with Gleason score.
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16 **CONCLUSIONS.** These results strongly suggested that CTEN plays an important role in paclitaxel
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18 sensitivity and [that CTEN](#) expression level may be a prognostic predictive factor for PCa patients.
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INTRODUCTION

Prostate cancer (PCa) is a major public health problem as it is the most commonly diagnosed cancer and the leading cause of cancer-related death in American men (1). Hormonal therapy (*i.e.*, androgen deprivation) initially induces [antitumor](#) response in more than 90% of patients. However, it eventually fails and the PCa progresses to an androgen-insensitive stage that is essentially incurable (2). Chemotherapy [plays](#) an increasingly important [role](#) in the management of androgen-insensitive metastatic PCa. Recently, taxanes (paclitaxel or docetaxel) in combination with other agents, such as estramustine phosphate, or [dexamethasone](#), for treating hormone-refractory PCa and have [been shown to induce](#) good [antitumor responses](#) (3–6). Paclitaxel acts as an antitumor drug by disrupting the cell cycle through stabilizing microtubule polymers (7). The microtubule cytoskeleton is a highly regulated system. At different times in the cell cycle, microtubules can be very stable or highly dynamic. Stability and dynamics are regulated by interaction with a large number of proteins that themselves [may](#) change at specific points in the cell cycle (8). Exogenous ligands [such as](#) paclitaxel can disrupt the normal processes by either increasing or decreasing microtubule stability and inhibiting their dynamic behavior (8).

Although hormone-resistant PCa initially responds to paclitaxel-based chemotherapy, [PCa](#) eventually becomes resistant to paclitaxel. One of main mechanisms of drug resistance is overexpression of the multiple drug resistance gene (MDR-1)-encoded P-glycoprotein, a drug transporter belonging to

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7 the ATP-binding cassette (9). Taxane_{resistance} has also been observed in several cancers. For example,
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10 in breast cancer, down-regulation of the gene encoding ribopholin II (RPN2) mediates docetaxel
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12 resistance by reducing glycosylation of P-glycoprotein (10). In ovarian cancers, overexpression of
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14 FOXO1 involving oxidative stress also contributes to drug_{resistance} (11). In pancreatic cancer,
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16 inhibition of BCL-2 alters diverse pathways that control cell survival and thus overcomes paclitaxel
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18 resistance (12).
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25 We have previously established paclitaxel-resistant DU145-TxR and PC-3-TxR cells from
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27 DU145 and PC-3 cell lines. In DU145 cells, paclitaxel resistance was due to overexpression of
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29 P-glycoprotein in DU145-TxR (13). However, in PC-3-TxR cells, knockdown of MDR-1 gene
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31 expression did not reverse paclitaxel_{resistance}, suggesting that other mechanisms are involved in
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33 paclitaxel_{resistance} of PC-3-TxR cells (13). Therefore, we performed cDNA microarray using mRNA
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35 from the parent cell lines PC-3 and PC-3-TxR and compared differentially expressed genes.
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Approximately 40000 genes were screened by cDNA microarray analysis. A total of 201 (1.34%) of the
screened genes were up-regulated by more than twofold, and 218 (1.45%) of the genes were
down-regulated by more than twofold in PC-3-TxR cells compared with PC-3 cells (13). We
hypothesized that some of these genes mediated paclitaxel_{resistance} in PC-3-TxR cells. We initially
focused on C-terminal tensin_{like} protein (CTEN), gene expression of which was down-regulated by
10-fold in PC-3-TxR cells (13).

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7 CTEN is a recently isolated focal adhesion molecule. Human CTEN cDNA encodes a
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10 715-amino acid sequence containing the Src homology 2 (SH2) and phosphotyrosine-binding (PTB)
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12 domains, [which](#) are similar to the COOH termini of tensin molecules that belong to [the four-member](#)
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14 tensin family ([tensin 1](#), [tensin 2](#), [tensin 3](#), and CTEN) ([14](#)). [The](#) proteins [encoded by these genes](#) are
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16 localized to the cytoplasmic side of focal adhesions ([14](#)). In the present study, we [examined whether](#)
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18 decreased CTEN expression contributes to paclitaxel resistance in PCa cells
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31 MATERIALS AND METHODS

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34 [Antibodies and reagents](#). The following primary antibodies were used: polyclonal anti-CTEN serum
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36 was raised by immunization of peptide 653-655 amino acid into a rabbit (Takara [Bio, Otsu](#), Japan),
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38 rabbit polyclonal anti-GAPDH, anti-actin, and anti-EGFR were purchased from Santa Cruz
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40 Biotechnology ([Santa Cruz](#), CA). Goat anti-rabbit IgG (H+L)-HRP Conjugate [was](#) purchased from
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42 Bio-Rad ([Hercules](#), CA). Paclitaxel (PTX) was purchased from ([Bristol Pharmaceuticals Y.K. Tokyo](#),
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44 [Japan](#)). Estramustine phosphate (EMP), docetaxel (DTX), doxorubicin (DOX), VP-16 (etoposide),
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46 [vinblastine](#) (VLB), [and](#) cisplatin (CDDP) were purchased from [Sigma](#) (St. Louis, MO). EGFR inhibitor
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48 PD153035 was purchased from Calbiochem (La Jolla, CA).
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59 **Cell lines and Cell culture.** Paclitaxel-resistant PC-3-TxR [and](#) DU145-TxR cells were [generated](#) and
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7 maintained as described previously (13). The PC-3-TxR cells were cultured in 10 nM paclitaxel to
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10 maintain their drug-resistant phenotypes. Before each experiment, these cells were grown for a
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12 minimum of one day in normal medium. The PC-3 and PC-3-TxR cells were maintained in RPMI1640
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15 (Sigma) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen,
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17 Carlsbad, CA). DU145 and DU145-TxR cells were maintained in Dulbecco's modified Eagle's medium
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19 (DMEM; Sigma) supplemented with 5% FBS.
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25 **Proliferation assay.** Cell growth or growth inhibition assay was performed by plating 2×10^5 cells on
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27 6-well plates. After cultured for 24 h, cells were treated with the indicated concentrations of anticancer
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29 agents (PTX, EMP, DTX, DOX, VP-16, VLB, and CDDP) or EGFR inhibitor PD153035 and cultured
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31 for an additional 48 h. At the end of the culture period, the cells were trypsinized and counted using a
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33 hemocytometer. The relative cell numbers compared with untreated controls were plotted as cell
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35 viability.
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43 **Plasmid transfection.** To generate a CTEN expression plasmid, the open reading frame of the CTEN
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45 gene was generated by RT-PCR using cDNA synthesized from PC-3 cells using the forward primer
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47 5'-ATCTCTGGGATGTCAGTGAGGCTGGTTG-3' and the reverse primer
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49 5'-GATGATGGTGACTGCTGAAGGCCATAGC-3'. After double digestion with *Xba*I and *Bam*HI, the
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51 PCR product was cloned into the respective restriction sites of the pBK-CMV-neo vector (Stratagene, La
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53 Jolla, CA). The insert was confirmed by sequencing from both directions, and the plasmid was named
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7 pBK-CMV-CTEN. PC-3-TxR and DU145-TxR cells were transfected with pBK-CMV-CTEN or
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10 pBK-CMV-neo using Lipofectamine reagent (Invitrogen, San Diego, CA) Eight h after transfection, the
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13 cells were cultured in medium containing 800 $\mu\text{g}/\text{mL}$ G418 (Sigma) and selected as stable
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16 CTEN-overexpressing cells.

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19 **Colony formation assay.** Cells were seeded at a density of 1.0×10^3 on 6-well plates, and allowed to
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22 adhere for 24 h. The cells were then treated with the indicated concentrations of paclitaxel, and medium
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25 was replaced with fresh medium after 24 h and every 3 days thereafter. The cells were allowed to grow
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28 for 10 days, then fixed using methanol and stained with 1% crystal violet, and the numbers of colonies
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31 containing >50 cells were counted. Treatment with each dose was performed in triplicate and the
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34 experiments were performed at least three times. The relative numbers of colonies compared with
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37 untreated controls were plotted as cell viability.

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40 **RNA interference analysis.** The specific Stealth CTEN and actin small interfering RNA (siRNA) were
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43 synthesized by Invitrogen. CTEN and actin target siRNA sequence were
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46 5'-AAUGUAGGAGUCAAGGUCCUCUGGG-3' and 5'-AUCUCUUUCUGCAUGCGGUCAGCGA-3',
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49 respectively. Validated Stealth EGFR siRNA and non-targeting siRNA (NT siRNA) were purchased
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52 from Invitrogen. For CTEN knockdown, PC-3, PC-3-TxR, DU145, and DU145-TxR cells were plated
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55 into 6-well plates at 3×10^5 cells/well, respectively. Cells were then transfected with 20 nM of CTEN
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58 siRNA or NT siRNA using X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN) for
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7 24 h. Total proteins were extracted 48 h after transfection. Twenty-four h after transfection with 20 nM
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10 NT siRNA or CTEN siRNA, cells were treated with [the](#) indicated concentrations of paclitaxel for 48 h,
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12 cultured for 48 h, and counted using a hemocytometer. For actin and EGFR knockdown, [24 h](#) after
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14 [transfection](#) with 20 nM of NT siRNA, actin, or EGFR siRNA, cells were treated with [the](#) indicated
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17 concentrations of paclitaxel for 48 h and counted.
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22 **Western blot analysis.** Twenty-four h after plating, total protein [was extracted](#) from PC-3, PC-3-TxR,
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24 DU145, and DU145-TxR cells as described previously (15). The subcellular protein (cytosol membrane
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26 nucleus and cytoskeleton protein) was extracted [using a](#) ProteoExtract Subcellular Proteome Extraction
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28 kit (Calbiochem). [Aliquots of](#) 30 µg [of](#) total [protein of](#) subcellular proteins were separated by 10%
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30 Ready Gel J (Bio-Rad), and electroblotted onto PVDF [membranes](#) (Bio-Rad), blocked with 5%
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32 skimmed milk, and reacted with anti-CTEN or rabbit polyclonal anti-GAPDH (Santa Cruz). The first
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34 antibody was recognized by goat anti-rabbit secondary antibody (Bio-Rad) and visualized using
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36 enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).
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47 **Immunofluorescence.** Staining for [tubulin](#), filamentous actin (F-actin) and CTEN protein was
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49 performed by overnight incubation [using commercial kits](#) in [accordance with the manufacturer's](#)
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51 [instructions \(Oregon Green® 488 conjugate kit, Phallotoxins and Zenon™ Tricolor Mouse and Rabbit](#)
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53 [IgG Labeling Kit; Molecular Probes, Eugene OR\)](#), Briefly, cells were fixed with 4% paraformaldehyde
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55 for 10 min, washed [three times](#) with PBS, and blocked with 5% bovine serum albumin (Sigma) for 15
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7 min. Slides were then washed three times with PBT (0.1% [Triton X-100](#) in PBS), [incubated](#) with
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9 anti-CTEN antibody for 1 h at 37°C, [and](#) cells [were washed](#) three times with PBT. The cells were [then](#)
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11 incubated with 5 µg/mL Alexa Flour 555 goat anti-rabbit IgG to detect anti-CTEN antibody in 1%
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13 BSA/PBT for 1 h at 37°C. The cells were also washed 3 times with PBT, and then incubated with
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15 [Oregon Green® 488 conjugate kit to detect tubulin and](#) Alexa Flour 488 phalloidin diluted 1:200 from
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17 stock solution for 1 h at 37°C to detect F-actin. The cells were washed 3 times with PBS, and mounted
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19 [with](#) Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) to detect nuclei. The
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21 slides were imaged using a confocal microscope.
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31 **Immunohistochemistry of tissue microarray.** PR951 [and PR952](#) tissue [microarrays](#) (TMA) [comprised](#)
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33 [of 176](#) cores [from 88](#) cases containing normal tissue, matched for Gleason score at surgery [were](#)
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35 [purchased](#) from Biomax (Rockville, MD). TMA sections were pretreated in 0.01 M sodium citrate buffer
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37 for 10 min in a microwave oven after [overnight incubation at 37°C](#). Endogenous peroxidase was
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39 blocked with 0.3% hydrogen peroxide, followed by incubation with PBS containing 10% normal goat
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41 serum. Specimens were incubated with anti-CTEN [antibody at](#) a dilution of 1:150. The antibody-antigen
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43 complex was visualized using the DakoCytomation LSAB+ system-HRP (Dako, [Carpinteria](#), CA). All
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45 sections were counterstained with [hematoxylin](#).
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56 **Statistical analysis.** [The](#) statistical significance of differences [in](#) proliferation [was determined by](#)
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58 [two-way ANOVA with post hoc](#) test. [Dunnett's test was](#) also performed [to determine the significance of](#)
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intensity differences on western blotting analysis. * $P < 0.05$ and ** $P < 0.01$ were considered statistically significant. Kruskal-Wallis test was used to determine the statistical significance of differences in immunohistochemical staining. The data represent the means \pm SD of three replicates.

RESULTS

Down-regulation of CTEN expression in paclitaxel-resistant PC-3 cells

In a previous study, we established paclitaxel-resistant PC-3 cells (PC-3-TxR) from androgen-independent PCa cells (PC-3). First, we investigated the expression level of α -tubulin and β -tubulin that form microtubules. There were no differences in their expression between PC-3 and PC-3-TxR cells (Fig. 1A). We also examined the distribution pattern of microtubules in these cells, but there were also no differences in distribution of microtubules in PC-3 and PC-3-TxR cells (Fig. 1B). Next, we reconfirmed paclitaxel resistance in PC-3-TxR cells using colony formation assay. PC-3-TxR cells were more resistant to paclitaxel than the parental PC-3 cells (LD_{50} : PC-3-TxR and PC-3, 30.2 nM and 2.0 nM, respectively) (Fig. 2A). To investigate which genes are responsible for paclitaxel resistance, we focused on those that were down-regulated in paclitaxel-resistant cells. Thus, we investigated the CTEN gene, which was down-regulated by 10-fold in PC-3-TxR cells compared with PC-3 cells (13). Western blot analysis showed that CTEN was strongly expressed in PC-3 cells but not in PC-3-TxR cells (Fig. 2A). To investigate whether down-regulation of CTEN expression occurred only during the

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7 process of establishment of PC-3-TxR cells, in which [the cells were grown](#) for a long period in
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9 paclitaxel, or whether paclitaxel treatment [rapidly](#) and directly affects CTEN expression, we treated
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11 PC-3 cells with paclitaxel and examined CTEN expression. Treatment with paclitaxel caused
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13 down-regulation of CTEN expression in PC-3 cells in a dose-dependent manner at 48 h (Fig. [2B](#)),
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16 indicating that paclitaxel can rapidly down-regulate CTEN expression. [Down-regulated CTEN](#)
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19 [expression in PC-3-TxR cells was irreversible even if we removed paclitaxel from the culture medium](#)
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22 [for maintenance of PC-3-TxR cells for at least 3 months \(data not shown\).](#)
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31 **Involvement of CTEN in paclitaxel sensitivity**

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34 We next [investigated whether](#) down-regulation of CTEN contributes to the development of
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36 paclitaxel resistance. [Then](#) we determined [the effect of](#) re-expression of CTEN [on](#) paclitaxel resistance
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38 in PC-3-TxR cells. We compared the sensitivity to paclitaxel between PC-3-TxR cells transfected with a
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40 CTEN expression vector (PC-3-TxR/CTEN) and those transfected with [empty](#) pBK-CMV-neo vector
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42 (PC-3-TxR/Neo). CTEN protein was detected at much higher [levels](#) in PC-3-TxR/CTEN compared to
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44 PC-3-TxR/Neo cells (Fig. [2C](#)). CTEN overexpression did not affect the proliferation of PC-3-TxR cells
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46 (Fig. [2C](#)). To investigate whether CTEN overexpression affects paclitaxel resistance, we compared
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48 paclitaxel sensitivity between PC-3-TxR/Neo and PC3-TxR/CTEN by colony [formation](#) assay. The
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50 survival curve for paclitaxel was shifted to [the](#) left by CTEN overexpression (LD₅₀ of PC-3-TxR/Neo
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7 and PC-3-TxR/CTEN: 15.2 nM and 4.5 nM, [respectively](#)) (Fig. [2D](#)), indicating that CTEN
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10 overexpression restored sensitivity to paclitaxel although the degree of restoration was not to the level of
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13 sensitivity observed in parental PC-3 cells (Fig. [2A](#)).
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16 To determine [whether a](#) decrease [in](#) CTEN expression [level](#) confers resistance to paclitaxel, we
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19 transfected PC-3 cells with CTEN siRNA or non-target (NT) siRNA. Transfection with CTEN siRNA
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22 repressed the expression of CTEN protein in PC-3 cells compared with NT siRNA (Fig. [2E](#)). PC-3 cells
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25 transfected with CTEN siRNA [showed greater resistance to paclitaxel](#) than PC-3 cells transfected with
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28 NT siRNA (LD₅₀ of PC-3/NT siRNA and PC-3/CTEN siRNA: 1.7 nM and 26.1 nM, respectively) (Fig.
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31 [2E](#)). These data [indicated](#) that [reduced](#) CTEN expression can induce paclitaxel [resistance](#) in PC-3 cells.
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38 **CTEN overexpression recovers paclitaxel sensitivity in other prostate cancer cells**

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40 We investigated whether CTEN overexpression affects paclitaxel sensitivity of other
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43 paclitaxel-resistant PCa cells as well as PC-3-TxR cells. Previously, we established paclitaxel [resistant](#)
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46 DU145 (DU145-TxR) cells in addition to PC-3-TxR cells ([13](#)). We first reconfirmed that DU145-TxR
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49 [cells](#) were resistant to paclitaxel compared to DU145 cells (Fig. [3A](#)). We had previously [shown](#) that
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52 increased expression of P-glycoprotein [contributes](#) to paclitaxel resistance of DU145-TxR cells ([13](#)).
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55 Both DU145 and DU145-TxR cells expressed [similarly](#) low levels of CTEN (Fig. [3A](#)). Therefore, we
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58 [examined whether](#) increased expression of CTEN could reverse paclitaxel resistance of DU145-TxR
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7 cells. These cells were stably transfected with pBK-CMV-CTEN (DU145-TxR/CTEN cell) or
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9 pBK-CMV-neo (DU145-TxR/Neo) (Fig. [3B](#)). Overexpression of CTEN did not affect cell proliferation
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11 of DU145-TxR cells (Fig. [3B](#)). Then, CTEN overexpression did not reduce P-glycoprotein levels in
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13 DU145-TxR/CTEN (Fig. 3C). However, CTEN overexpression partially restored paclitaxel sensitivity
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17 (Fig. [3D](#), compare with DU145 in Fig. [3A](#)), suggesting that mechanisms other than P-glycoprotein were
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21 involved in restoration of paclitaxel sensitivity by CTEN.
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28 **CTEN overexpression partly recovers the sensitivity to other anti-tumor drugs**

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31 We also compared the cross-resistance to other anticancer drugs, i.e., DTX (docetaxel), VBL
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33 (vinblastine), VP-16 (etoposide), CDDP (cisplatin), DOX (doxorubicin), and EMP (estramustine
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35 phosphate), between PC-3-TxR/Neo and PC-3-TxR/CTEN cells. CTEN overexpression restored the
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37 sensitivity to DTX, which belongs to the taxane family similar to paclitaxel (Fig. [4](#)). CTEN
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39 overexpression also partially restored the sensitivity for CDDP, VP-16, VBL, DOX, and EMP,
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41 suggesting that CTEN affects the sensitivity to different anticancer drugs through a common pathway
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47 although the main mechanisms of drug resistance are different among these drugs (Fig. [4](#)).
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56 **Mechanisms of paclitaxel resistance by down-regulation of CTEN**

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59 To investigate the mechanisms by which decreased CTEN expression promotes paclitaxel
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7 resistance in PCa cells, we first examined the [differences](#) in expression level of apoptosis-related
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10 proteins because paclitaxel initiates the apoptotic process by binding to [b-tubulin](#) and promoting its
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12 polymerization [\(16\)](#). We observed no [differences](#) in expression of [a-tubulin](#), [b-tubulin](#), caspase 3, 7, 8, 9,
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15 10, bcl-2, bcl-xL, [or](#) bax proteins among PC-3, PC-3-TxR, PC-3-TxR/Neo, and PC-3-TxR/CTEN cells
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17 by western [blotting](#) analysis (data not shown). These results [suggested](#) that [alterations of the](#) apoptotic
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19 response do not account for the development of paclitaxel sensitivity.
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25 We next examined the localization of CTEN protein in PC-3 cells. [The results of](#) western
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27 [blotting](#) analysis of various subcellular fractions [indicated that CTEN protein was localized mainly at](#)
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29 [the cytoskeleton in PC-3 cells](#) (Fig. [5A](#)). Immunofluorescence analysis showed that CTEN expression
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31 was down-regulated by treatment with paclitaxel as [shown](#) in Fig. [1C](#) (Fig. [5B](#)). [As](#) CTEN was localized
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33 at [the](#) cytoskeleton [similar to](#) other tensins, we investigated the [effects](#) of paclitaxel on the expression of
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35 F-actin, [which](#) is [also](#) localized at [the](#) cytoskeleton. This analysis [indicated](#) that F-actin was up-regulated
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37 by paclitaxel in PC-3 cells (Fig. [5B](#)). To [determine](#) whether the [effects](#) of paclitaxel on expression of
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39 F-actin in PC-3 cells [are](#) due to the [changes in](#) CTEN expression induced by paclitaxel, we compared
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41 CTEN expression with F-actin expression in PC-3, PC-3-TxR, PC-3-TxR/Neo, and PC-3-TxR/CTEN
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43 cells. [Immunofluorescence](#) analyses of CTEN and F-actin revealed [an](#) inverse correlation between
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45 CTEN and F-actin expression among these [cell lines](#) (Fig. [5C](#)). Moreover, knockdown of CTEN in PC-3
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47 cells by CTEN siRNA transfection induced F-actin expression (Fig. [5C](#)). To confirm whether
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7 down-regulation of actin expression changed paclitaxel sensitivity, we knocked down actin expression
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10 by transfection [of actin siRNA](#) into PC-3-TxR cells and examined paclitaxel sensitivity. Knockdown of
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12 actin partially restored paclitaxel sensitivity (Fig. [5D](#)). These results suggest that one of [the](#) mechanisms
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14 through which paclitaxel resistance is induced by down-regulation of CTEN expression is associated
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16 with elevation of actin, [which](#) is localized [to](#) the same region as CTEN.
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25 **Another mechanism of paclitaxel resistance by down-regulation of CTEN**

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28 Recently, several groups demonstrated that epidermal growth factor receptor (EGFR) is
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30 involved in paclitaxel resistance. Paclitaxel-resistant cells expressed [higher levels of](#) EGFR, and EGFR
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32 tyrosine kinase inhibitor was more effective in resistant cells than in paclitaxel-sensitive cells (17–20).
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35 [Therefore, we postulated](#) that CTEN [may affect](#) EGFR expression and modulate paclitaxel sensitivity.
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38 To explore this possibility, we compared the expression of EGFR between PC-3 and PC-3-TxR cells.
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41 EGFR expression was elevated [to a greater extent](#) in PC-3-TxR cells than in [the parental](#) PC-3 [cell line](#)
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43 (Fig. [6A](#)). To confirm the effect of CTEN on EGFR expression, we compared EGFR expression
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45 between PC-3-TxR/Neo and PC-3-TxR/CTEN cells. Overexpression of CTEN in PC-3-TxR
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47 down-regulated EGFR expression (Fig. [6A](#)). [In addition](#), we knocked down CTEN in PC-3, which
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49 resulted in up-[regulation of](#) EGFR expression (Fig. [6A](#)). Having determined that CTEN inversely
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51 regulates EGFR expression, we next evaluated whether EGFR expression affects paclitaxel sensitivity.
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7 Knockdown of EGFR expression by transfection [of EGFR siRNA](#) into PC-3-TxR cells restored
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10 paclitaxel sensitivity (Fig. [6B](#)). Next, we investigated the effects of [CTEN knockdown in PC-3 and](#)
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12 [CTEN overexpression in PC-3-TxR on sensitivity to the EGFR inhibitor PD153035](#). There were no
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14 [differences in sensitivity to PD153035 regardless of the increase or decrease of CTEN expression and](#)
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16 [EGFR expression in these cells](#). These data suggested that PD153035 has the same effect on these cells
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19 [as long as EGFR is expressed \(Fig. 6C\)](#). We also examined whether [PD153035](#) affected paclitaxel
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21 sensitivity in PC-3-TxR cells. [Administration of 1 μM PD153035, which](#) did not affect proliferation of
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23 PC-3-TxR cells ([Fig. 6C](#)), diminished paclitaxel resistance in PC-3-TxR cells (Fig. [6D](#)). These results
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26 [indicated](#) that overexpression of EGFR induced by down-regulation of CTEN mediates paclitaxel
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28 resistance in PC-3-TxR cells.
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41 **CTEN protein expression correlates with Gleason Score and metastasis in prostate cancer**

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43 To [examine](#) whether the CTEN protein [is](#) differentially expressed in PCa tissues compared to
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45 benign tissues, immunohistochemical staining was performed on tissue microarray specimens comprised
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47 from 89 cores [from](#) 44 cases containing normal tissue. All specimens were graded using the Gleason
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49 score. CTEN was differentially expressed in PCa specimens and non-neoplastic tissues (Fig. [7](#) and Table
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51 1). [In](#) non-neoplastic tissues, [15](#) of [16 \(94%\)](#) expressed [high](#) CTEN, [25](#) of [28 \(89%\)](#) Gleason score 6 [or](#)
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53 7 PCa tissue samples [showed](#) high [CTEN](#) expression [level](#), [6](#) of [12 \(50%\)](#) Gleason score 8 PCa tissues
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7 [showed](#) intermediate expression of CTEN, and [26](#) of [32](#) (81%) [Gleason score 9 or 10](#), PCa tissues
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10 [showed](#) low or no expression of CTEN. [Positive](#) staining [for CTEN](#) was located mostly in epithelial
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12 cells, but was also noted in some extracellular areas surrounding neoplastic glands and epithelial cells.
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16 This study showed that CTEN protein expression was inversely correlated with pathological Gleason
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18 scores of PCa ([P<0.001](#)); CTEN protein was down-regulated in poorly differentiated PCa tissue.
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28 DISCUSSION

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31 Although hormone-refractory PCa initially respond to taxanes, eventually the PCa develops
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33 resistance to the taxanes and progresses to end stage [disease](#). Therefore, it is extremely important to
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35 understand the mechanisms [by](#) which PCa [becomes](#) resistant to taxanes to overcome the development of
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37 taxane resistance. The strategy to determine [the](#) mechanisms that contribute to taxane resistance is to
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39 identify genetic or epigenetic aberrations underlying sensitivity/resistance. One mechanism of paclitaxel
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41 resistance is overexpression of P-glycoprotein, [the effect of](#) which [is mediated by](#) pumping taxanes out
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43 of the cell [\(9\)](#). However, [this](#) mechanism is not always [applicable](#) to all cells. Although PC-3-TxR cells
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45 have increased [levels of](#) P-glycoprotein expression, knockdown of P-glycoprotein had no impact on
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47 paclitaxel resistance indicating that P-glycoprotein does not mediate paclitaxel resistance in PC-3-TxR
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49 cells [\(13\)](#). [Therefore, we](#) explored other mechanisms of paclitaxel resistance and [showed](#) that
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7 down-regulation of CTEN/tensin 4 induces paclitaxel_{resistance} in PC-3-TxR cells. Moreover,
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10 overexpression of CTEN not only in PC-3-TxR cells but also in DU145-TxR cells, in which
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12 overexpression of P-glycoprotein was the main reason for paclitaxel_{resistance} (13), restored paclitaxel
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14 sensitivity. Furthermore, overexpression of CTEN partly restored sensitivity to other drugs (DTX,
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16 CDDP, VP-16, EMP, DOX, and VBL). Previously, we confirmed cross-resistance of PC-3-TxR cells for
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18 these drugs except CDDP and VP-16 (13). At that time, we could not clarify the mechanism through
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20 which PC-3-TxR became resistant to these drugs. The results of the present study suggested that the
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22 reduced expression of CTEN may be a common mechanism of drug resistance and that CTEN
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24 overexpression by some strategies, such as gene therapy, may improve chemosensitivity regardless of
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26 CTEN expression in cancer cells.
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37 CTEN is a recently identified focal adhesion molecule that is specifically expressed in the
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39 prostate (14). CTEN belongs to the four-member tensin family, the proteins belonging to which are
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41 localized to the cytoplasm of focal adhesions (14). Tensin 1, the prototype of the family, interacts with
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43 actin filaments in multiple ways (21), and contains an Src homology 2 (SH2) domain that binds to
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45 phosphotyrosine-containing proteins (22,23). CTEN (C-terminal tensin_{like}) is a distant member of the
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47 family with a smaller molecular mass than the others. CTEN shows homology to other tensin family
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49 members through the presence of the SH2 and PTB domains but it does not have the actin-binding
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51 domain found in other tensin family members (14). The function of CTEN in the cytoskeleton, if any,
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7 remains unknown.
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10 In the [present study, we showed that](#) modulation of CTEN expression inversely affects
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12 paclitaxel resistance. Due to the role of tensins in the cytoskeleton, we [examined whether](#) alteration of
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14 CTEN expression [had an impact on](#) cytoskeletal proteins. Although CTEN does not have an
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16 actin-binding domain, down-regulation of CTEN in PC-3-TxR cells induced F-actin expression. The
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18 cytoskeleton is crucial for many cellular processes. For example, [the](#) function of cytoskeletal F-actin is
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20 linked to the invasive and metastatic phenotypes of malignant cancer cells (24,25). The cytoskeleton is
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22 composed of intermediate filaments, microfilaments, microtubules, the microtrabecular lattice, and
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24 other structures characterized by a polymeric filamentous nature and long-range order within the cell.
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26 The various elements of the cytoskeleton not only serve in the maintenance of cellular shape but also
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28 have roles in other cellular functions, including cellular movement, cell division, endocytosis, apoptosis,
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30 and movement of organelles [\(26 – 29\)](#). Cytoskeletal proteins provide the structural foundation that
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32 allows cells to exist in a highly organized [state \(30\)](#). These reports suggest that elevation of F-actin by
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34 CTEN down-regulation may modify the cytoskeletal cell structure to confer resistance to paclitaxel.
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50 [Similar to F-actin, we](#) also confirmed that paclitaxel resistance caused by CTEN
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52 down-regulation was partially mediated through elevation of EGFR expression. Moreover, EGFR
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54 tyrosine kinase inhibitor restored paclitaxel sensitivity in PC-3-TxR cells. Kitazaki *et al.* showed that an
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56 EGFR tyrosine kinase inhibitor directly inhibited the function of P-glycoprotein in multidrug-resistant
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7 cancer cells (31). However, there seems to be little interaction between P-glycoprotein and EGFR in
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10 PC-3-TxR cells as paclitaxel resistance of PC-3-TxR cells was not involved in P-glycoprotein in our
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13 previous study (13). Recently, Pu *et al.* showed that the EGFR inhibitor PD168393 potentiated the
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16 cytotoxic effects of paclitaxel synergistically with Bad, p53, and p21^{Waf1/Cip1} induction and ERK1/2
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19 inactivation (32). Coley *et al.* demonstrated that ERK-phosphorylation and survivin were involved in
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22 EGFR activation in drug-resistant cells (18). These data suggest that combination therapy with taxanes
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25 and EGFR tyrosine kinase inhibitors will provide new strategies to overcome paclitaxel resistance. Our
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28 findings suggest that CTEN may be an upstream target to inhibit EGFR activity and thus may be worthy
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31 of further exploration for inhibition of drug resistance.

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34 Paclitaxel down-regulated CTEN expression within 48 h. Little is known about how CTEN
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37 expression is regulated by paclitaxel. Liao *et al.* demonstrated that b-catenin up-regulated CTEN
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40 expression in colon cancer (33). However, we found no differences in b-catenin expression among PC-3,
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43 PC3-TxR, DU145, and DU145-TxR cells. We are currently investigating the mechanism of regulation of
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46 CTEN by paclitaxel.

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50 Although we did not observe a difference in cell proliferation between PC-3-TxR/Neo and
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53 PC-3-TxR/CTEN *in vitro*, CTEN expression was inversely associated with PCa Gleason score. Our
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56 findings were in agreement with those of a previous report that CTEN expression was lower in PCa than
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59 in the normal prostate (14). In contrast, CTEN mRNA expression was correlated with tumor progression
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7 in lung [and colon cancer \(33,34\)](#). This discrepancy could be due to the [differences in](#) tissue type. [In](#)
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10 [addition](#), the CTEN gene localizes to chromosome 17q21, a region frequently deleted in PCa [\(35,36\)](#).
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13 Furthermore, due to tissue differences, the function of CTEN as a focal adhesion molecule may be
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16 different among different cancer tissues. Regardless, our results suggest that [the](#) expression level of
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19 CTEN could be a biomarker of PCa progression. [In addition](#), the observation that only 60% of men with
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22 androgen non-responsive PCa respond to initial taxane therapy indicates that a large number of PCa
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25 patients are initially resistant to taxanes. If we could predict the responsiveness [to](#) taxanes prior to
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28 chemotherapy, we could avoid [administration of](#) unnecessary and toxic taxane-based treatment regimens.
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31 Our results suggest that evaluation of CTEN expression in PCa tissues may be a useful way to predict
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34 taxane responsiveness. Unfortunately, it is extremely difficult to obtain recurrent samples from [patients](#)
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37 before chemotherapy because the recurrence [is](#) often in bone [metastatic lesions and](#) not in the prostate.
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40 [We](#) are now [collecting data from HRPC patients treated with taxanes and will investigate the](#) correlation
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43 between CTEN expression [at](#) diagnosis and duration [of](#) taxanes responsiveness.
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47 In conclusion, we [showed](#) that down-regulation of CTEN causes paclitaxel [resistance](#) in PCa
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50 cells. This was associated with elevation of F-actin and increased EGFR, [which](#) contributed to this
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53 resistance. Moreover, expression of CTEN was [inversely](#) correlated with Gleason Score, indicating that
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56 poorly differentiated PCa may have increased resistance to taxane-based [therapy](#). Accordingly, defining
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59 the function and regulation of CTEN may lead to new chemotherapy strategies for those patients
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7 initially resistant or that [later](#) develop resistance to taxanes.
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REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA: a cancer journal for clinicians* 2008;58(2):71-96.
2. Gopalkrishnan RV, Kang DC, Fisher PB. Molecular markers and determinants of prostate cancer metastasis. *J Cell Physiol* 2001;189(3):245-256.
3. Obasaju C, Hudes GR. Paclitaxel and docetaxel in prostate cancer. *Hematology/oncology clinics of North America* 2001;15(3):525-545.
4. Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr., Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *The New England journal of medicine* 2004;351(15):1513-1520.
5. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *The New England journal of medicine* 2004;351(15):1502-1512.
6. Oudard S, Banu E, Beuzeboc P, Voog E, Dourthe LM, Hardy-Bessard AC, Linassier C, Scotte F, Banu A, Coscas Y, Guinet F, Poupon MF, Andrieu JM. Multicenter randomized phase II study of

1
2
3
4
5
6
7 two schedules of docetaxel, estramustine, and prednisone versus mitoxantrone plus prednisone in
8
9 patients with metastatic hormone-refractory prostate cancer. *J Clin Oncol*
10
11 2005;23(15):3343-3351.
12
13

- 14
15
16 7. Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule
17
18 dynamics, drug transport, and cell death. *J Clin Oncol* 1999;17(3):1061-1070.
19
20
21 8. Downing KH. Structural basis for the interaction of tubulin with proteins and drugs that affect
22
23 microtubule dynamics. *Annual review of cell and developmental biology* 2000;16:89-111.
24
25
26 9. Fojo T, Menefee M. Mechanisms of multidrug resistance: the potential role of
27
28 microtubule-stabilizing agents. *Ann Oncol* 2007;18 Suppl 5:v3-8.
29
30
31 10. Honma K, Iwao-Koizumi K, Takeshita F, Yamamoto Y, Yoshida T, Nishio K, Nagahara S, Kato
32
33 K, Ochiya T. RPN2 gene confers docetaxel resistance in breast cancer. *Nature medicine*
34
35 2008;14(9):939-948.
36
37
38 11. Goto T, Takano M, Hirata J, Tsuda H. The involvement of FOXO1 in cytotoxic stress and
39
40 drug-resistance induced by paclitaxel in ovarian cancers. *Br J Cancer* 2008;98(6):1068-1075.
41
42
43 12. Mortenson MM, Galante JG, Gilad O, Schlieman MG, Virudachalam S, Kung HJ, Bold RJ.
44
45 BCL-2 functions as an activator of the AKT signaling pathway in pancreatic cancer. *Journal of*
46
47 cellular biochemistry 2007;102(5):1171-1179.
48
49
50 13. Takeda M, Mizokami A, Mamiya K, Li YQ, Zhang J, Keller ET, Namiki M. The establishment
51
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6
7 of two paclitaxel-resistant prostate cancer cell lines and the mechanisms of paclitaxel resistance
8
9
10 with two cell lines. *Prostate* 2007;67(9):955-967.
- 11
12
13 14. Lo SH, Lo TB. Cten, a COOH-terminal tensin-like protein with prostate restricted expression, is
14
15
16 down-regulated in prostate cancer. *Cancer Res* 2002;62(15):4217-4221.
- 17
18
19 15. Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, Honma S, Keller ET, Namiki M.
20
21
22 The adrenal androgen androstenediol is present in prostate cancer tissue after androgen
23
24
25 deprivation therapy and activates mutated androgen receptor. *Cancer Res* 2004;64(2):765-771.
- 26
27
28 16. Stein CA. Mechanisms of action of taxanes in prostate cancer. *Semin Oncol* 1999;26(5 Suppl
29
30
31 17):3-7.
- 32
33
34 17. Dai Q, Ling YH, Lia M, Zou YY, Kroog G, Iwata KK, Perez-Soler R. Enhanced sensitivity to the
35
36
37 HER1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib hydrochloride in
38
39
40 chemotherapy-resistant tumor cell lines. *Clin Cancer Res* 2005;11(4):1572-1578.
- 41
42
43 18. Coley HM, Shotton CF, Ajose-Adeogun A, Modjtahedi H, Thomas H. Receptor tyrosine kinase
44
45
46 (RTK) inhibition is effective in chemosensitising EGFR-expressing drug resistant human ovarian
47
48
49 cancer cell lines when used in combination with cytotoxic agents. *Biochem Pharmacol*
50
51
52 2006;72(8):941-948.
- 53
54
55 19. Qiu L, Di W, Jiang Q, Scheffler E, Derby S, Yang J, Kouttab N, Wanebo H, Yan B, Wan Y.
56
57
58 Targeted inhibition of transient activation of the EGFR-mediated cell survival pathway enhances
59
60

- 1
2
3
4
5
6
7 paclitaxel-induced ovarian cancer cell death. *Int J Oncol* 2005;27(5):1441-1448.
- 8
9
10 20. Nozawa H, Tadakuma T, Ono T, Sato M, Hiroi S, Masumoto K, Sato Y. Small interfering RNA
11 targeting epidermal growth factor receptor enhances chemosensitivity to cisplatin, 5-fluorouracil
12 and docetaxel in head and neck squamous cell carcinoma. *Cancer science*
13 2006;97(10):1115-1124.
- 14
15
16
17
18
19 21. Lo SH, Janmey PA, Hartwig JH, Chen LB. Interactions of tensin with actin and identification of
20 its three distinct actin-binding domains. *The Journal of cell biology* 1994;125(5):1067-1075.
- 21
22
23
24
25
26
27 22. Davis S, Lu ML, Lo SH, Lin S, Butler JA, Druker BJ, Roberts TM, An Q, Chen LB. Presence of
28 an SH2 domain in the actin-binding protein tensin. *Science (New York, NY)*
29 1991;252(5006):712-715.
- 30
31
32
33
34
35
36
37 23. Cui Y, Liao YC, Lo SH. Epidermal growth factor modulates tyrosine phosphorylation of a novel
38 tensin family member, tensin3. *Mol Cancer Res* 2004;2(4):225-232.
- 39
40
41
42
43
44 24. Greiner S, Humrich JY, Thuman P, Sauter B, Schuler G, Jenne L. The highly attenuated vaccinia
45 virus strain modified virus Ankara induces apoptosis in melanoma cells and allows bystander
46 dendritic cells to generate a potent anti-tumoral immunity. *Clinical and experimental*
47 immunology 2006;146(2):344-353.
- 48
49
50
51
52
53
54
55
56 25. Vignjevic D, Montagnac G. Reorganisation of the dendritic actin network during cancer cell
57 migration and invasion. *Seminars in cancer biology* 2008;18(1):12-22.
- 58
59
60

- 1
2
3
4
5
6
7 26. Vicente-Manzanares M, Sanchez-Madrid F. Role of the cytoskeleton during leukocyte responses.
8
9 Nature reviews 2004;4(2):110-122.
10
11
12 27. Shih YL, Rothfield L. The bacterial cytoskeleton. Microbiol Mol Biol Rev 2006;70(3):729-754.
13
14
15 28. Herrmann H, Aebi U. Intermediate filament assembly: fibrillogenesis is driven by decisive
16
17 dimer-dimer interactions. Current opinion in structural biology 1998;8(2):177-185.
18
19
20 29. Kamal A, Goldstein LS. Connecting vesicle transport to the cytoskeleton. Current opinion in cell
21
22
23 biology 2000;12(4):503-508.
24
25
26
27 30. Lo SH, Weisberg E, Chen LB. Tensin: a potential link between the cytoskeleton and signal
28
29
30 transduction. Bioessays 1994;16(11):817-823.
31
32
33 31. Kitazaki T, Oka M, Nakamura Y, Tsurutani J, Doi S, Yasunaga M, Takemura M, Yabuuchi H,
34
35
36 Soda H, Kohno S. Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of
37
38
39 P-glycoprotein in multidrug resistant cancer cells. Lung cancer (Amsterdam, Netherlands)
40
41
42 2005;49(3):337-343.
43
44
45
46 32. Pu YS, Hsieh MW, Wang CW, Liu GY, Huang CY, Lin CC, Guan JY, Lin SR, Hour TC.
47
48
49 Epidermal growth factor receptor inhibitor (PD168393) potentiates cytotoxic effects of paclitaxel
50
51
52 against androgen-independent prostate cancer cells. Biochem Pharmacol 2006;71(6):751-760.
53
54
55 33. Liao YC, Chen NT, Shih YP, Dong Y, Lo SH. Up-regulation of C-terminal tensin-like molecule
56
57
58 promotes the tumorigenicity of colon cancer through beta-catenin. Cancer Res
59
60

1
2
3
4
5
6
7 2009;69(11):4563-4566.

- 8
9
10 34. Sasaki H, Moriyama S, Mizuno K, Yukiue H, Konishi A, Yano M, Kaji M, Fukai I, Kiriyama M,
11 Yamakawa Y, Fujii Y. Cten mRNA expression was correlated with tumor progression in lung
12 cancers. Lung cancer (Amsterdam, Netherlands) 2003;40(2):151-155.
13
14
15
16
17
18
19 35. Gao X, Zacharek A, Grignon DJ, Sakr W, Powell IJ, Porter AT, Honn KV. Localization of
20 potential tumor suppressor loci to a < 2 Mb region on chromosome 17q in human prostate cancer.
21 Oncogene 1995;11(7):1241-1247.
22
23
24
25
26
27
28 36. Williams BJ, Jones E, Zhu XL, Steele MR, Stephenson RA, Rohr LR, Brothman AR. Evidence
29 for a tumor suppressor gene distal to BRCA1 in prostate cancer. J Urol 1996;155(2):720-725.
30
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Legends

Figure 1. Cellular microtubule structures of PC-3 and PC-3-TxR cells. (A) Western blotting analysis of a-tubulin and b-tubulin protein expression. (B) Tubulin polymerization of PC-3 and PC-3-TxR cells. Tubulins were stained with an Oregon Green® 488 conjugate kit (green) and DAPI (blue). PC-3-TxR cells exhibited similar tubulin polymerization (Green) to the parental PC-3 cell line.

Figure 2. Down-regulation of CTEN expression is related to paclitaxel resistance in PC-3 cells. (A) CTEN expression and paclitaxel sensitivity in PC-3 and PC-3-TxR cells. Total proteins extracted from untreated PC-3 and PC-3-TxR cells were subjected to western blotting analysis of CTEN and GAPDH. Anti-CTEN antibody and anti-GAPDH antibody were used for detection of 76 kD CTEN and 37 kD GAPDH protein, respectively. Colony formation assay was performed as described in Materials and Methods. (B) Regulation of CTEN expression by paclitaxel. Western blotting analysis of CTEN was performed after treatment of PC-3 cells with paclitaxel for 48 h. The relative intensity compared with untreated PC-3 cells was columned. (C) Proliferation of PC-3-TxR/Neo and PC-3-TxR/CTEN cells. The numbers of PC-3-TxR/Neo and PC-3-TxR/CTEN cells were counted 24, 48, 72, and 96 h after inoculation of 2×10^3 cells. NS: no significant difference. (D) Sensitivity of PC-3-TxR/Neo and

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7 PC-3-TxR/CTEN cells. Total proteins extracted from PC-3-TxR/Neo and PC-3-TxR/CTEN cells were
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10 subjected to western [blotting](#) analysis of CTEN and GAPDH. Colony formation assay of PC-3-TxR/Neo
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12 and PC-3-TxR/CTEN cells after treatment with paclitaxel for 24 h (A). (E) knockdown of CTEN
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14 expression in PC-3 cells by CTEN siRNA transfection. [Twenty-four h after transfection](#) with NT siRNA
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16 or CTEN siRNA, total proteins from PC-3 cells were extracted and subjected to western [blotting](#)
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18 analysis of CTEN and GAPDH. Growth inhibition by paclitaxel was [examined](#) after transfection with
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20 NT siRNA (PC-3/NT siRNA) or CTEN siRNA (PC-3/CTEN siRNA) as described in Materials and
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35 **Figure 3.** Overexpression of CTEN increases sensitivity to paclitaxel in DU145-TxR cells. (A) Total
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37 [proteins](#) from DU145 and DU145-TxR cells were subjected to western [blotting](#) analysis for CTEN and
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39 GAPDH. Colony formation assay of DU145 and DU145-TxR cells were performed as described in
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41 Materials and Methods. (B) Total proteins from DU145-TxR/Neo and DU145-TxR/CTEN cells were
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43 subjected to western [blotting](#) analysis of CTEN and GAPDH. DU145-TxR/Neo and DU145-TxR/CTEN
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45 [cell proliferation were](#) compared after [inoculation of \$2 \times 10^4\$ cells](#). NS: no significant difference. (C)
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47 [Comparison of P-glycoprotein expression among DU145, DU145-TxR, DU145-TxR/Neo, and](#)
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49 [DU145-TxR/CTEN.](#) (D) Colony formation [assays](#) of DU145-TxR/Neo and DU145-TxR/CTEN cells
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51 [were](#) performed as described in Fig. 1.
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10 **Figure 4.** Comparison of sensitivity [to several drugs](#) between PC-3-TxR/Neo and PC-3-TxR/CTEN
11 cells. PC-3-TxR/Neo and PC-3-TxR/CTEN cells were exposed to the indicated concentrations of DTX,
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13 CDDP, VP-16, VLB, EMP, and DOX for 24 h and [the](#) numbers of the cells were counted 48 h after
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17 exposure.
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25 **Figure 5.** Localization of CTEN protein and involvement of actin [in](#) paclitaxel resistance. (A) The
26 subcellular protein fractions (cytoplasm, membrane, nucleus, and cytoskeleton protein) were extracted
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28 as described in Materials and Methods and subjected to western [blotting](#) analysis for CTEN. (B)
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32 Immunofluorescence analysis of CTEN and F-actin after treatment [with](#) paclitaxel. After PC-3 cells
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34 were treated with or without paclitaxel (30 nM) for 24 h, immunofluorescence [analyses](#) were performed
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36 using rabbit anti-CTEN antibody (red), F-actin (green) as described in Materials and [Methods, and the](#)
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38 blue signal represents nuclear DNA staining (400×[_](#)magnification). (C) Immunofluorescence analysis of
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CTEN and F-actin in PC-3, PC-3-TxR, PC-3-TxR/Neo, and PC-3-TxR/CTEN cells and PC-3
transfected with NT siRNA or CTEN siRNA. Immunofluorescence analysis was performed as [described](#)
[in](#) (B). (D) [Effects](#) of actin expression on paclitaxel sensitivity. PC-3-TxR cells transfected with NT
siRNA (20 nM) or actin siRNA (5, 10, or 20 nM) for 24 h were [subjected to](#) western [blotting](#) analysis of
actin and GAPDH. Anti-actin antibody and anti-GAPDH antibody were employed for detection of 43

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7 [kD](#) actin and 37 [kD](#) GAPDH protein, respectively. PC-3-TxR cells transfected with 20 nM NT or actin
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10 siRNA for 24 h were treated with paclitaxel for 24 h. Then, the cells were cultured for 48 h in normal
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12 medium.

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19 **Figure 6. Effects** of CTEN on EGFR expression and involvement of EGFR for paclitaxel resistance. (A)
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21 Western [blotting](#) analysis of EGFR. Total proteins from PC-3, PC-3-TxR, PC-3-TxR/Neo, and
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23 PC-3-TxR/CTEN were subjected to western [blotting](#) analysis. Total proteins from PC-3/NT siRNA, and
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25 PC-3-TxR/CTEN were also subjected to western [blotting](#) analysis. Anti-CTEN, anti-EGFR, [and](#)
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27 anti-GAPDH [antibodies](#) were employed for detection of CTEN, EGFR, [and](#)
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29 anti-GAPDH [antibodies](#) were employed for detection of CTEN, EGFR, [and](#) GAPDH, respectively. (B)
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31 [Effects](#) of EGFR siRNA on paclitaxel sensitivity. Twenty-four h after [transfection of](#) PC-3-TxR cells
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33 with 20 nM NT siRNA or EGFR siRNA, the cells were treated with paclitaxel for 24 h. Then, the cells
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35 were cultured for 48 h in normal medium. (C) [Effects of EGFR inhibitor PD153035 on cell viability of](#)
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37 [PC-3/NT siRNA and PC-3/CTEN siRNA. Twenty-four h after transfection of PC-3 cells with 20 nM NT](#)
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39 [siRNA or CTEN siRNA, the cells were treated with the indicated concentration of PD153035 for 48 h](#)
40
41 [and the numbers of cells were counted.](#) (D) [Effects](#) of EGFR inhibitor PD153035 on paclitaxel
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43 sensitivity. PC-3-TxR cells were treated with paclitaxel with or without 1 μ M PD153035 for 48 h and
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45 the numbers of cells were counted.
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7 **Figure 7.** Immunohistochemistry of CTEN in prostate tissue. Representative examples of
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9 photomicrographs (40× and 200× magnification) showing CTEN expression in [the](#) normal prostate and
10 prostate cancer on tissue microarray [analysis](#). (A) CTEN expression [in](#) normal prostate tissue (intensity
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16 +++). (B) CTEN expression in prostate cancer with Gleason score 7 (intensity ++). (C) CTEN
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18 expression in prostate cancer with Gleason score 8 (intensity +). (D) CTEN expression in prostate
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22 cancer with Gleason score 9 (intensity [-](#)).
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TABLE 1. Immunohistochemistry of CTEN in normal prostate and prostate cancer tissue on tissue microarray analysis

Clinicopathological features		CTEN Expression				Total Number
		(-)	(+)	(++)	(+++)	
Normal		0	1	2	13	16
Gleason score	6, 7	0	3	11	14	28
	8	2	4	5	1	12
	9, 10	11	15	4	2	32
Total number		13	23	22	30	88

Fig. 1 Li et al.

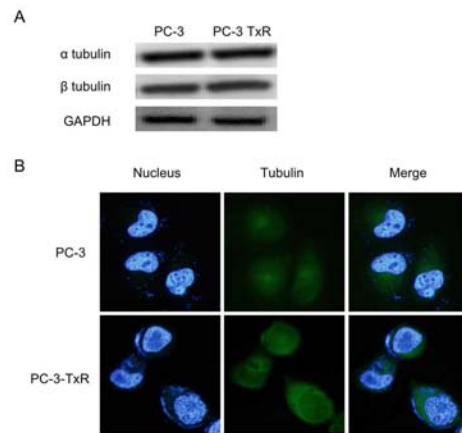


Figure 1. Cellular microtubule structures of PC-3 and PC-3-TxR cells. (A) Western blotting analysis of α -tubulin and β -tubulin protein expression. (B) Tubulin polymerization of PC-3 and PC-3-TxR cells. Tubulins were stained with an Oregon Green® 488 conjugate kit (green) and DAPI (blue). PC-3-TxR cells exhibited similar tubulin polymerization (Green) to the parental PC-3 cell line.
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Fig. 2 Li et al.

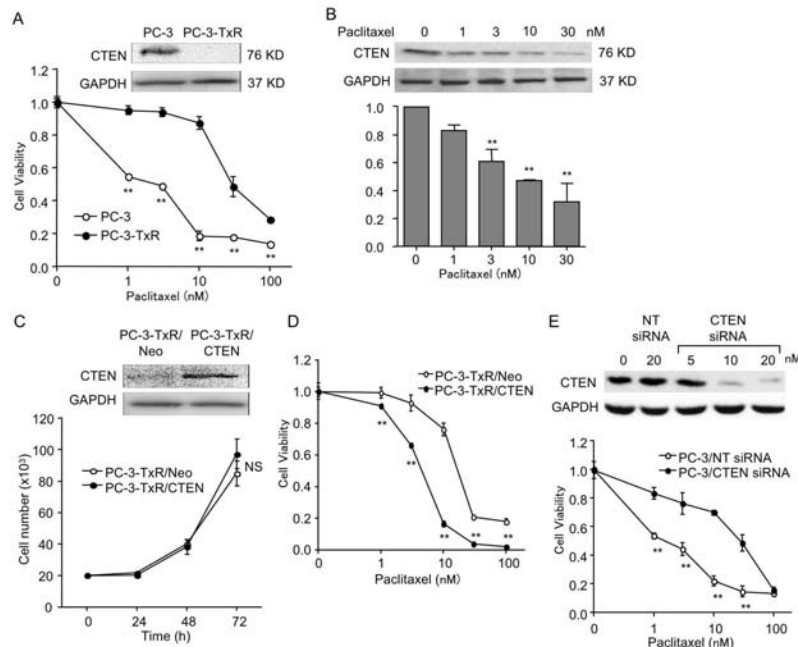


Figure 2. Down-regulation of CTEN expression is related to paclitaxel resistance in PC-3 cells. (A) CTEN expression and paclitaxel sensitivity in PC-3 and PC-3-TxR cells. Total proteins extracted from untreated PC-3 and PC-3-TxR cells were subjected to western blotting analysis of CTEN and GAPDH.

Anti-CTEN antibody and anti-GAPDH antibody were used for detection of 76 kD CTEN and 37 kD GAPDH protein, respectively. Colony formation assay was performed as described in Materials and Methods. (B) Regulation of CTEN expression by paclitaxel. Western blotting analysis of CTEN was performed after treatment of PC-3 cells with paclitaxel for 48 h. The relative intensity compared with untreated PC-3 cells was columned. (C) Proliferation of PC-3-TxR/Neo and PC-3-TxR/CTEN cells. The numbers of PC-3-TxR/Neo and PC-3-TxR/CTEN cells were counted 24, 48, 72, and 96 h after inoculation of 2×10^3 cells. NS: no significant difference. (D) Sensitivity of PC-3-TxR/Neo and PC-3-TxR/CTEN cells. Total proteins extracted from PC-3-TxR/Neo and PC-3-TxR/CTEN cells were subjected to western blotting analysis of CTEN and GAPDH. Colony formation assay of PC-3-

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3 TxR/Neo and PC-3-TxR/CTEN cells after treatment with paclitaxel for 24 h (A). (E) knockdown of
4 CTEN expression in PC-3 cells by CTEN siRNA transfection. Twenty-four h after transfection with NT
5 siRNA or CTEN siRNA, total proteins from PC-3 cells were extracted and subjected to western
6 blotting analysis of CTEN and GAPDH. Growth inhibition by paclitaxel was examined after
7 transfection with NT siRNA (PC-3/NT siRNA) or CTEN siRNA (PC-3/CTEN siRNA) as described in
8 Materials and Methods.
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Fig. 3 Li et al.

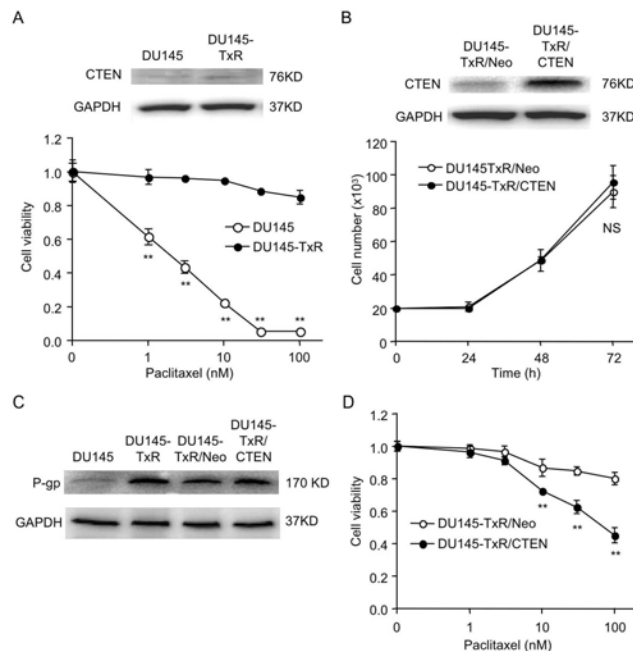


Figure 3. Overexpression of CTEN increases sensitivity to paclitaxel in DU145-TxR cells. (A) Total proteins from DU145 and DU145-TxR cells were subjected to western blotting analysis for CTEN and GAPDH. Colony formation assay of DU145 and DU145-TxR cells were performed as described in Materials and Methods. (B) Total proteins from DU145-TxR/Neo and DU145-TxR/CTEN cells were subjected to western blotting analysis of CTEN and GAPDH. DU145-TxR/Neo and DU145-TxR/CTEN cell proliferation were compared after inoculation of 2×10^4 cells. NS: no significant difference. (C) Comparison of P-glycoprotein expression among DU145, DU145-TxR, DU145-TxR/Neo, and DU145-TxR/CTEN. (D) Colony formation assays of DU145-TxR/Neo and DU145-TxR/CTEN cells were performed as described in Fig. 1.

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Fig. 4 Li et al.

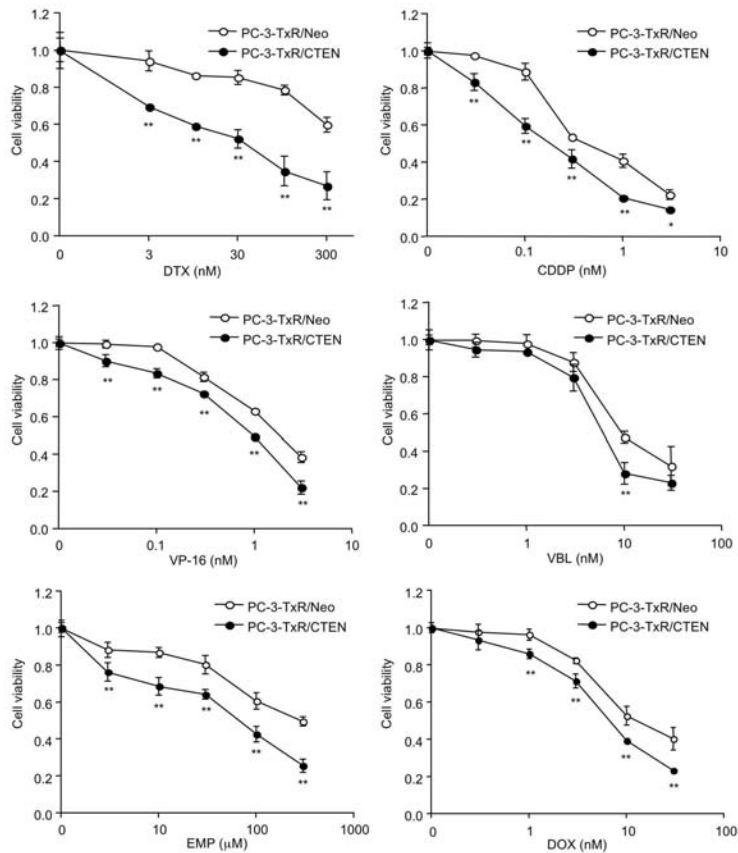


Figure 4. Comparison of sensitivity to several drugs between PC-3-TxR/Neo and PC-3-TxR/CTEN cells. PC-3-TxR/Neo and PC-3-TxR/CTEN cells were exposed to the indicated concentrations of DTX, CDDP, VP-16, VLB, EMP, and DOX for 24 h and the numbers of the cells were counted 48 h after exposure.

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Fig. 5 Li et al.

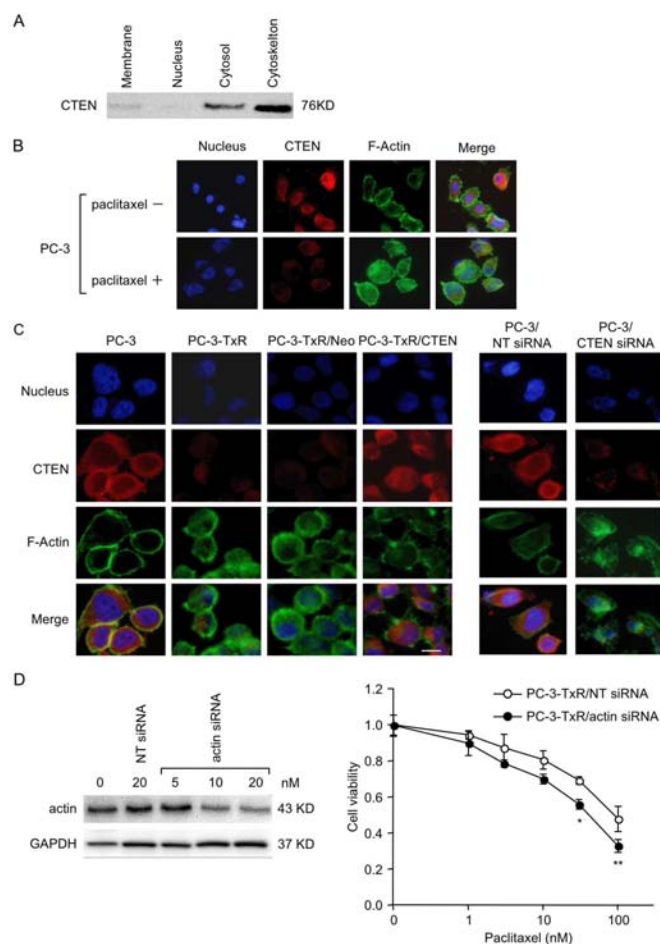


Figure 5. Localization of CTEN protein and involvement of actin in paclitaxel resistance. (A) The subcellular protein fractions (cytoplasm, membrane, nucleus, and cytoskeleton protein) were extracted as described in Materials and Methods and subjected to western blotting analysis for CTEN. (B) Immunofluorescence analysis of CTEN and F-actin after treatment with paclitaxel. After PC-3 cells were treated with or without paclitaxel (30 nM) for 24 h, immunofluorescence analyses were performed using rabbit anti-CTEN antibody (red), F-actin (green) as described in Materials and Methods, and the blue signal represents nuclear DNA staining (400 \times magnification). (C) Immunofluorescence analysis of CTEN and F-actin in PC-3, PC-3-TxR, PC-3-TxR/Neo, and PC-3-TxR/CTEN cells and PC-3 transfected with NT siRNA or CTEN siRNA. Immunofluorescence analysis was performed as described in (B). (D) Effects of actin expression on paclitaxel sensitivity. PC-3-TxR cells transfected with NT siRNA (20 nM) or actin siRNA (5, 10, or 20 nM) for 24 h were subjected to western blotting analysis of actin and GAPDH. Anti-actin antibody and anti-GAPDH

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antibody were employed for detection of 43 kD actin and 37 kD GAPDH protein, respectively. PC-3-TxR cells transfected with 20 nM NT or actin siRNA for 24 h were treated with paclitaxel for 24 h. Then, the cells were cultured for 48 h in normal medium.
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Fig. 6 Li et al.

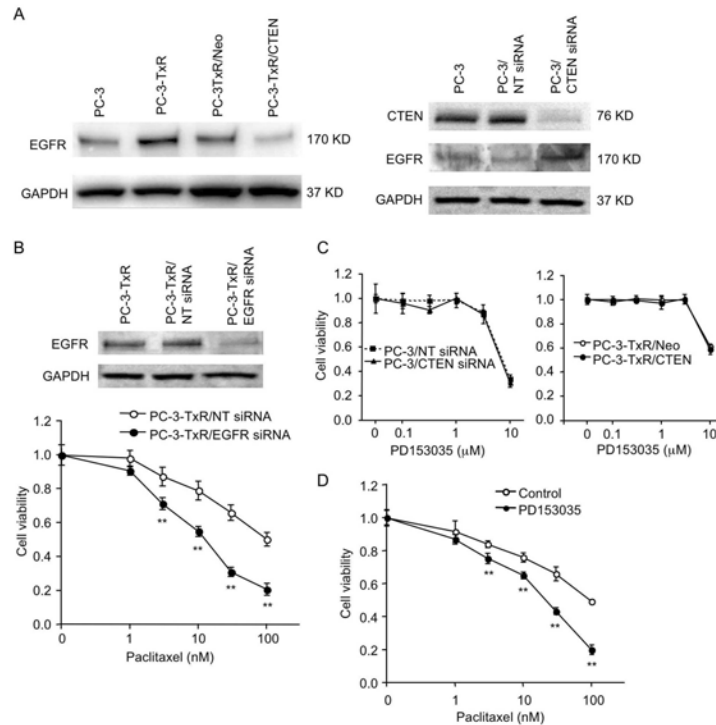


Figure 6. Effects of CTEN on EGFR expression and involvement of EGFR for paclitaxel resistance. (A) Western blotting analysis of EGFR. Total proteins from PC-3, PC-3-TxR, PC-3-TxR/Neo, and PC-3-TxR/CTEN were subjected to western blotting analysis. Total proteins from PC-3/NT siRNA, and PC-3/CTEN siRNA were also subjected to western blotting analysis. Anti-CTEN, anti-EGFR, and anti-GAPDH antibodies were employed for detection of CTEN, EGFR, and GAPDH, respectively. (B) Effects of EGFR siRNA on paclitaxel sensitivity. Twenty-four h after transfection of PC-3-TxR cells with 20 nM NT siRNA or EGFR siRNA, the cells were treated with paclitaxel for 24 h. Then, the cells were cultured for 48 h in normal medium. (C) Effects of EGFR inhibitor PD153035 on cell viability of PC-3/NT siRNA and PC-3/CTEN siRNA. Twenty-four h after transfection of PC-3 cells with 20 nM NT siRNA or CTEN siRNA, the cells were treated with the indicated concentration of PD153035 for 48 h and the numbers of cells were counted. (D) Effects of EGFR inhibitor PD153035 on paclitaxel sensitivity. PC-3-TxR cells were treated with paclitaxel with or without 1 μM PD153035 for 48 h and

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the numbers of cells were counted.
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Fig. 7 Li et al.

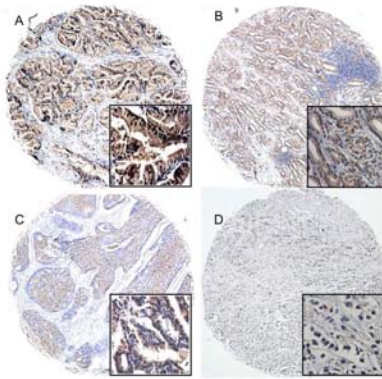


Figure 7. Immunohistochemistry of CTEN in prostate tissue. Representative examples of photomicrographs (40 \times and 200 \times magnification) showing CTEN expression in the normal prostate and prostate cancer on tissue microarray analysis. (A) CTEN expression in normal prostate tissue (intensity +++). (B) CTEN expression in prostate cancer with Gleason score 7 (intensity ++). (C) CTEN expression in prostate cancer with Gleason score 8 (intensity +). (D) CTEN expression in prostate cancer with Gleason score 9 (intensity -).
199x266mm (300 x 300 DPI)